Simultaneous visual assessment of RNA and protein expression in circulating tumor cells using the AccuCyte-CyteFinder System

Abstract 3789

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Background

Circulating tumor cells (CTCs) provide real-time information regarding patient tumor phenotype, including RNA expression. This information may be valuable in guiding care of cancer patients. Simultaneous RNA and protein assessment has been reported previously in large populations of cells. Here, we describe methods to simultaneously evaluate RNA and protein expression on rare single CTCs using the AccuCyte – CyteFinder system (RareCyte).

Methods

To establish RNA detection in model CTCs (mCTCs), we used LNCaP and SkBr3 cells spiked into blood. Isolated buffy coat was processed by AccuCyte onto microscope slides. RNAscope® in-situ hybridization (ISH, Advanced Cell Diagnostics) was performed to detect Her2, Pan-CTC, AR, AR-v7, UBC (positive control), POLR2A (positive control), and dapB (negative control) expression. A process to simultaneously stain for RNA and protein was developed to allow identification of mCTCs by protein expression of cytokeratin (CK) and EpCAM and measurement of gene expression with RNAscope. Various blood collection tubes were tested to measure gene expression, protein expression, and cell recovery 24 hours after blood draw. Image analysis software was developed to automatically analyze and count RNA dot number from 40x z-stack images of mCTCs (Fig. 3). Finally, clinical samples were stained with the combined RNA/protein assay.

Results

Using RNAscope, Her2, POLR2A, and UBC expression could be detected in SkBr3 and LNCaP cells and was negative in surrounding white blood cells (WBC) (Figs. 1, 2). An RNA/protein assay was compared using blood collected and stored in EDTA, CellSave®, Cell-Free DNA®, Cell-Free RNA®, Cyto-Chex®, and RareCyte BCT tubes (Table 1). At 48 hours, Cell-Free RNA tubes had the highest number of RNA dots/cell – about the same as fresh EDTA samples (Table 3). Using blood collected into EDTA tubes and processed immediately after spike-in, SkBr3 cells had an average of 29 Her2 mRNA dots and 20 UBC dots/cell; negative control dapB gave 1 dot/cell (Fig. 4 and Table 2). Using CK and EpCAM expression for mCTC identification, recovery of SkBr3 cells was 95% (Table 4). Clinical samples were successfully stained with the RNA/protein assay (Fig. 5).

Conclusions

We have developed a protocol that identifies rare CTCs using protein staining, and measures RNA expression by RNAscope ISH. This assay may be a useful clinical tool for the real-time investigation of CTC gene expression in cancer patients.

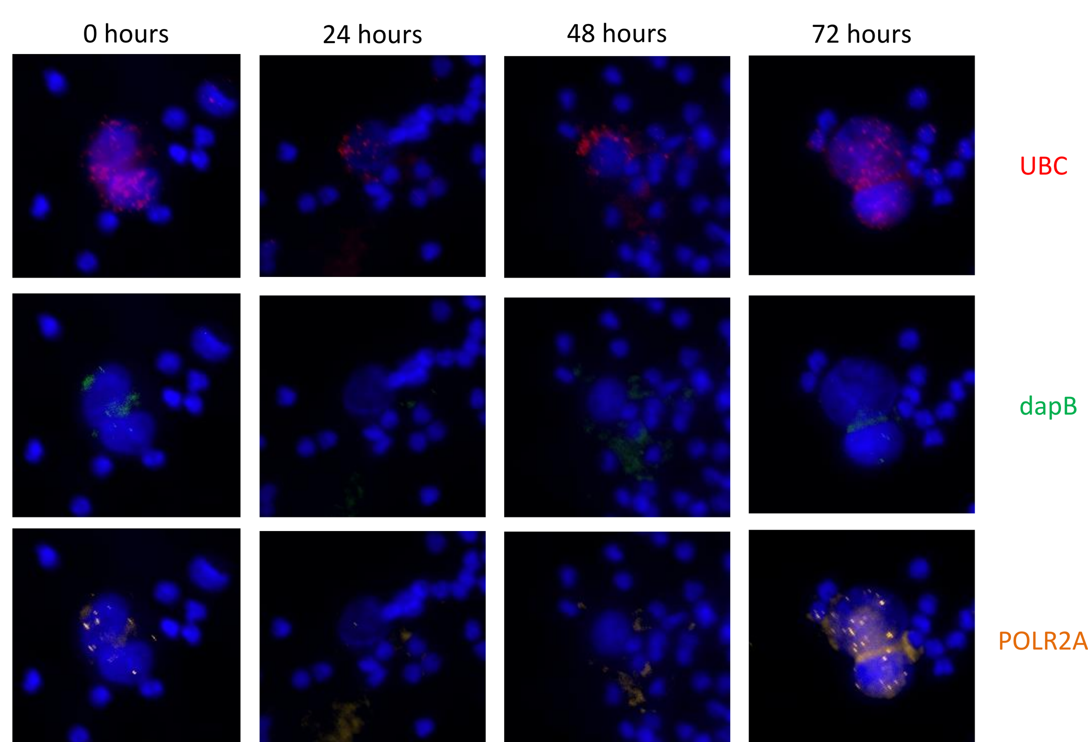

Figure 1. Blood was collected into an EDTA tube, LNCaP cells were spiked in, and blood was transferred to a cfRNA tube. Each day, an aliquot was taken out and processed. After all samples were processed, RNAscope was performed in parallel to measure RNA degradation over time.

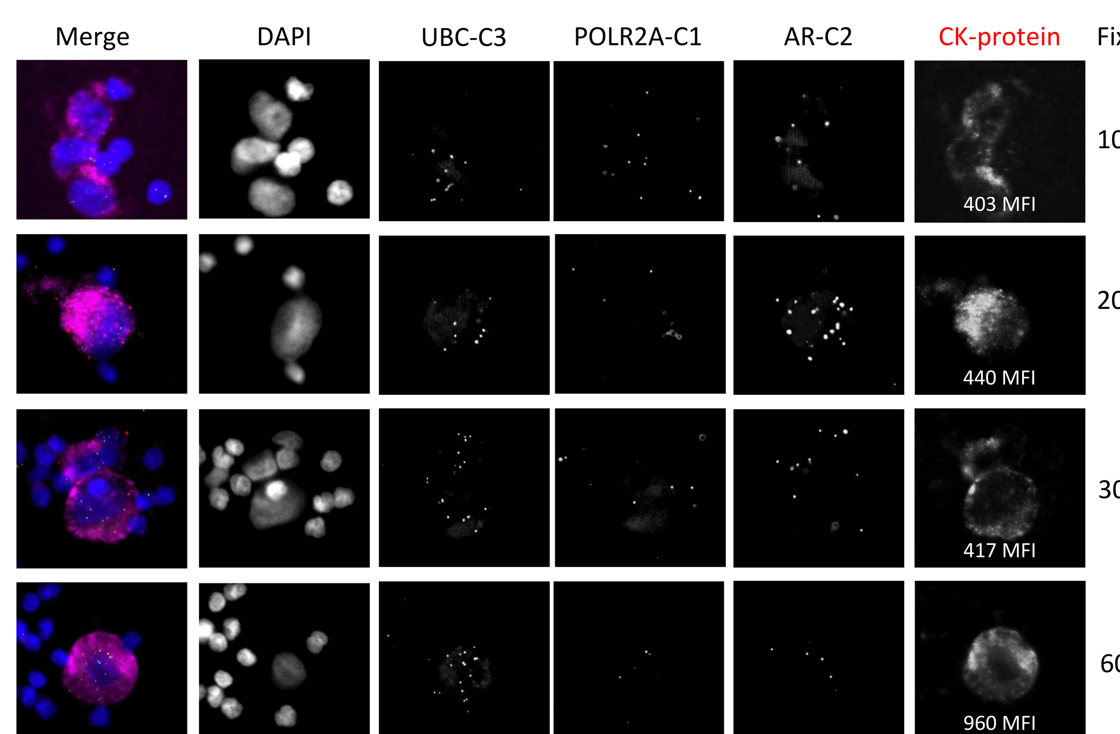

Figure 2. Samples were processed as in figure 1. Slides from 0 hours were fixed for different amounts of time in 10% NBF. RNAscope was performed, followed by manual staining for CK using a secondary antibody conjugated to QD-800 to detect signal. MFI=mean fluorescence intensity.

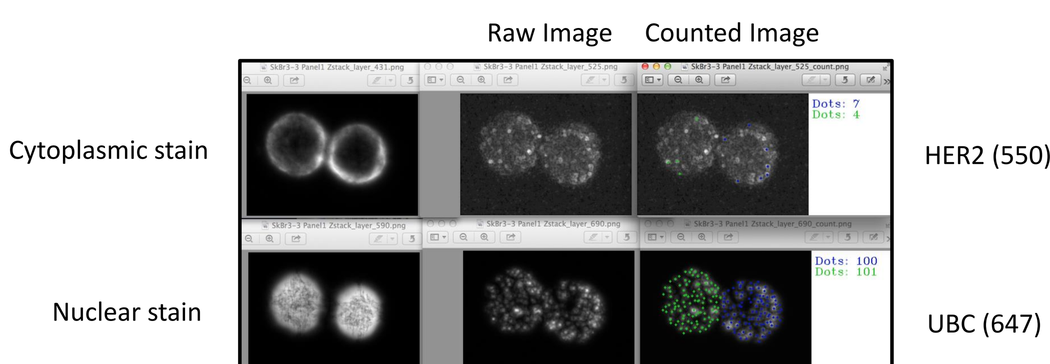

Figure 3. RNA dot counter program uses a mask in the cytoplasmic channel to find CTCs among WBC, a nuclear mask to identify number of CTCs in a cluster, and a mask in the RNA channel(s) to count the RNA dots per cell on a single image or a Z-stack.

Tube	Recovery (%)	UBC +	UBC -	UBC %	dots/cell	Comments
RareCyte	72	6	4	60%	12.9	Very good CK staining, UBC on many WBC
EDTA	39	7	4	64%	12	Lower CK staining, UBC mostly on CTCs
cfRNA	21	4	2	67%	17.7	Good CK staining, UBC mostly on CTCs
cfDNA	48	10	2	83%	10.3	Very good CK staining, UBC sometimes on WBC
CytoChex	24	NA	NA	NA	NA	Recovery of CTCs too low to quantify
CellSave	18	NA	NA	NA	NA	Recovery of CTCs too low to quantify

Table 1. Several blood collection tubes were processed 24 h after collection and spike in of LNCaP cells. CK and EpCAM protein stain was evaluated (Leica stainer, no antigen retrieval) followed by RNAscope for UBC. Recovery of CTCs (before RNAscope), UBC staining, and protein stain were quantified.

Condition	# of HER2 RNA dots	# of UBC RNA dots	# of dapB RNA dots	Average # of dots		
				HER2	UBC	dapB
EDTA-**0h**	19, 18, 16, 25, 23, 18, 28, 33, 10, 22, 14, 11, 14, 26, 24, 16, 36, 38, 28, 39, 37, 21, 18, 20, 22, 28, 35, 45, 31, 46, 34, 58, 23, 20, 23, 29, 67, 29, 27, 33, 37, 32, 37, 28, 29, 44, 30, 12, 39, 49, 32, 45, 26, 18, 27, 25, 18, 12, 49, 40, 57, 27, 0, 46, 31, 6, 8	28, 25, 16, 17, 25, 25, 23, 23, 19, 19, 18, 11, 29, 21, 21, 39, 20, 13, 20, 26, 26, 10, 18, 23, 30, 19, 4, 30, 12, 10, 12	1, 1, 1, 1, 0, 0, 0, 0, 1, 1, 3, 2, 1, 4, 0, 0, 0, 0, 1, 4, 2, 6, 4, 2, 1, 1, 3,	**29**	20.4	1.4
EDTA-**48h**	11, 9, 7, 4, 3, 4, 2, 4, 0, 1, 1, 5, 2, 13, 13, 11, 4, 4, 11, 7, 4, 8, 10, 6, 2, 1, 1, 3, 6, 18, 4, 6, 1, 0, 11, 9, 3, 6, 16, 12, 10, 7, 2, 14, 5, 4, 3, 9, 1, 9, 2	9, 35, 15, 3, 5, 27, 8, 3, 8, 9, 4, 7, 1, 0, 10, 15, 18, 4, 7, 2, 0, 3, 9, 6, 4, 19, 10, 10, 3, 14, 29, 8, 6, 6, 5, 5, 10, 8	0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0	**5.9**	9.1	0

Table 2. SkBr3 cells were spiked into EDTA blood, an aliquot was processed right away, another was left for 48 h. Slides were stained for RNAscope, followed by manual staining as in figure 2.

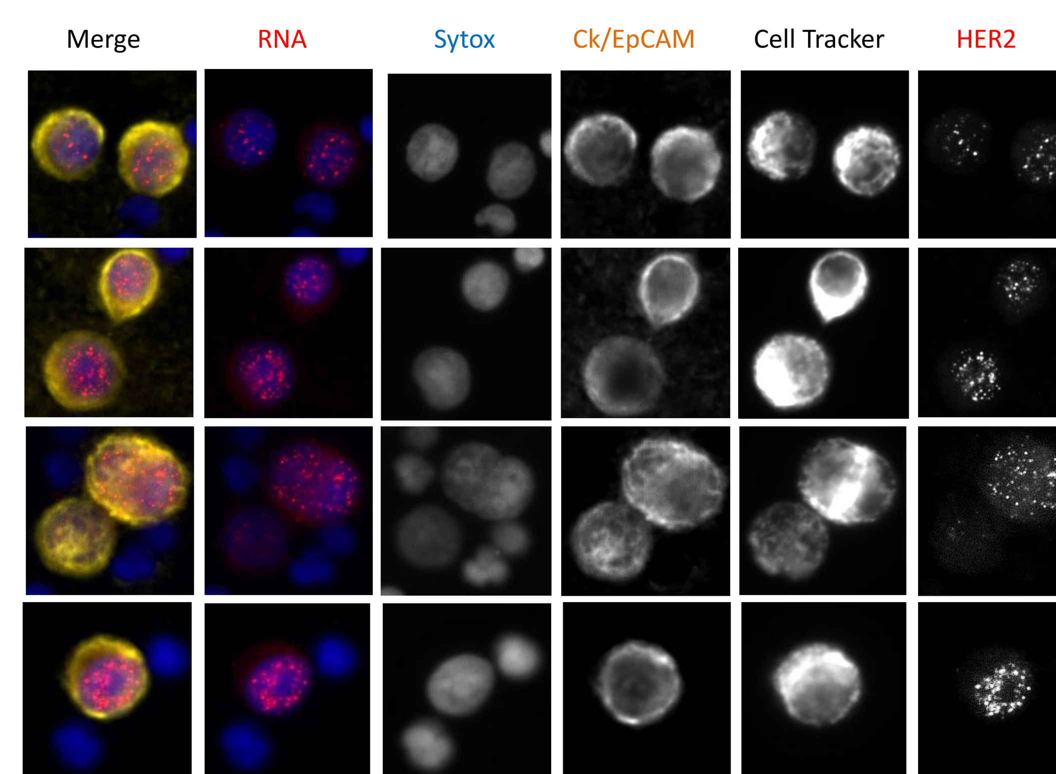

Figure 4. Images of SkBr3 cells spiked into EDTA blood and processed at 0 h.

Slide	# of UBC RNA dots	Average
cfRNA-0h	2, 4, 5, 3, 0, 5, 4, 6, 8, 3, 4, 1, 1, 1, 2, 14, 9, 1, 1	3.9
cfRNA-48h	29, 15, 8, 22, 44, 17, 25, 0, 0, 23, 13	17.8

Table 3. Blood was collected into EDTA tube, SkBr3 cells were spiked in, and blood was transferred to cfRNA tube. An aliquot was processed right away, another was left for 48 h. Slides were stained for RNAscope, followed by manual staining for CK as in figure 2.

Slide	CTC recovery	Percent recovery
EDTA-0h	114/100	114%
EDTA-0h	99/100	99%
EDTA-48h	75/100	75%
EDTA-48h	92/100	92%
EDTA-48h	94/100	94%

Table 4. Sample was processed as in Table 2. Recovery was measured after RNAscope and CK staining .

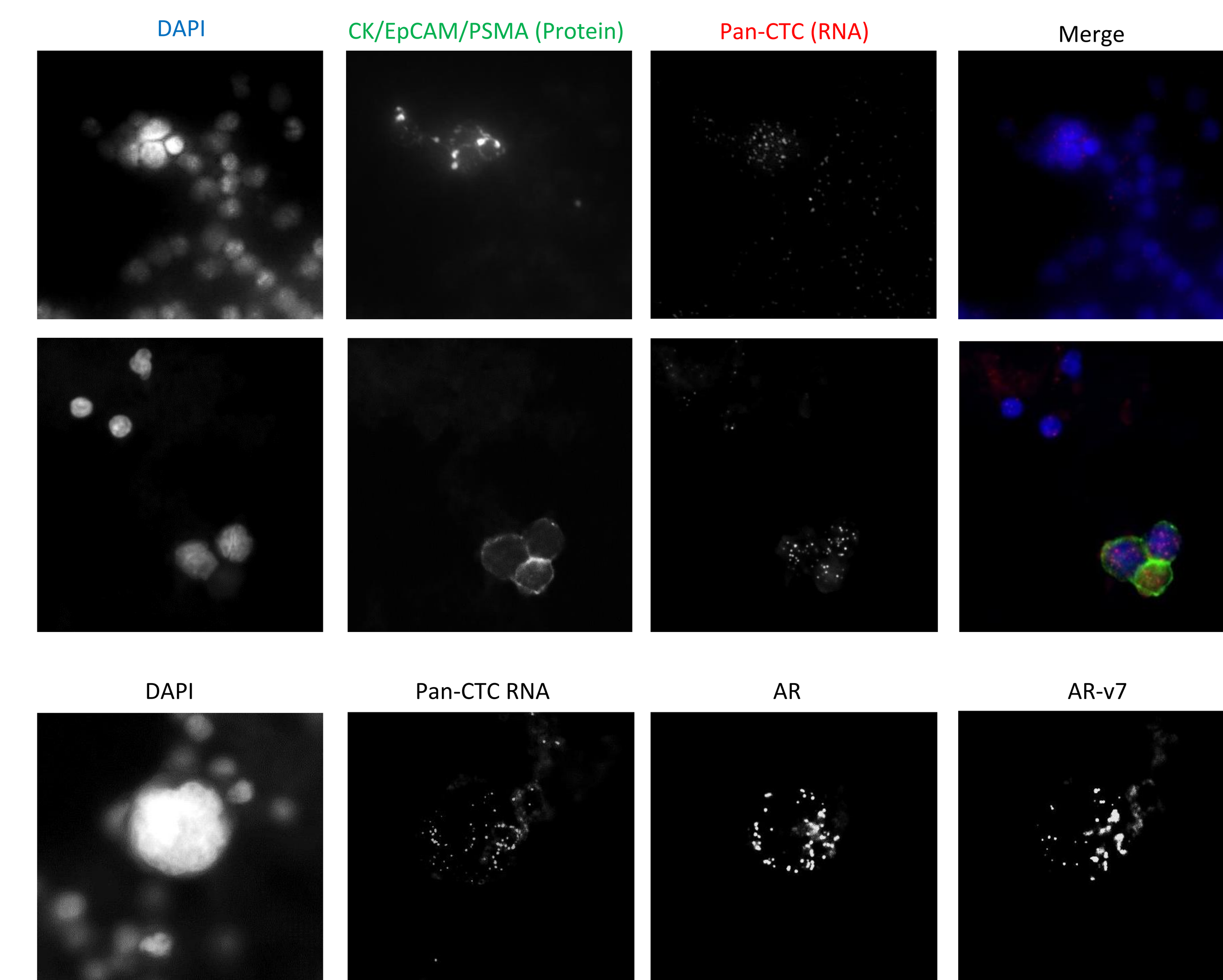

Figure 5. First row: blood from a triple negative breast cancer patient was collected into EDTA, processed 24 hours later. RNAscope was performed for a Pan-CTC marker panel (KRT8, 14, 17, 18, 19, 20; MUC1; EPCAM; FN1; CDH2), followed by manual CK/EpCAM/PSMA protein stain. Recovery was estimated at 30% compared to a non-RNAscope control slide. Second row: blood from a prostate cancer patient was collected into EDTA, processed right away. RNAscope was performed as in first row. Recovery was estimated at 15% compared to a non-RNAscope control slide. Third row: sample from second row was stained with RNAscope for AR and AR-v7.